Towards Improved Therapeutic CORMs: Understanding the Reactivity of CORM-3 with Proteins

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Abstract: The biological role of carbon monoxide (CO) has completely changed in the last decade. Beyond its widely feared toxicity, CO has revealed a very important biological activity as a signaling molecule with marked protective actions namely against inflammation, apoptosis and endothelial oxidative damage. Its direct use as a therapeutic gas showed significant and consistent positive results but also intrinsic severe limitations. The possibility of replacing the gas by pro-drugs acting as CO-Releasing Molecules (CO-RMs) has clearly been demonstrated with several experimental compounds. Transition metal carbonyls complexes have proven to be the most versatile experimental CO-RMs so far. Presently, the challenge is to equip them with drug-like properties to turn them into useful pharmaceuticals. This requires studying their interactions with biological molecules namely those that control their pharmacokinetic and ADME profiles like the plasma proteins. In this account we analyze these questions and review the existing interactions between Metal Carbonyls and proteins. The recently explored case of CORM-3 is revisited to exemplify the methodologies involved and the importance of the results for the understanding of the mode of action of such pro-drugs.

Keywords: CO therapy, CO-RM, CORM-3, Hen Egg White Lysozyme, protein-metal adduct, metal carbonyl complex, X-ray crystallography.

CO: A THERAPEUTICALLY ACTIVE SMALL MOLECULE

Endogenously produced in living organisms as a product of heme catabolism, CO is now recognized as a gaseotransmitter together with NO and H_2S [1, 2]. CO is involved in multiple defense mechanisms in normal physiologic processes and is overproduced in pathologic situations, namely inflammation [3, 4]. In agreement with its protective signalling functions, CO inhaled in doses well below toxic levels acts as a strong anti-inflammatory and anti-apoptotic agent, prevents endothelial damage due to various oxidative stresses, and actively promotes endothelial healing [3]. These and other significant and consistent therapeutic effects observed in many animal models of disease led to the on-going clinical trials of CO gas therapy in kidney transplantation, post operative ileus and chronic obstructive pulmonary disease [5].

In its gaseous form, however, CO presents serious limitations as a drug, both for administration in a clinical setting and, in particular, for the use by patients outside the hospital. Because it is a gas, CO would have to be administered with the help of a mask, or other cumbersome equipment and accurate dosing could present a problem for the patient. This is a major obstacle for the use of CO in human therapy, in particular for chronic diseases where treatment could be required frequently or over long periods of time. Another, equally important difficulty is the fact that, after inhalation, CO binds with high affinity to hemoglobin, forming a carboxyhemoglobin (COHb) complex and thus not reaching the tissues effectively. Consequently, in order to exert its therapeutic effect on specific tissues in need, e.g. sites of inflammation, gaseous CO typically causes significant increases in COHb levels in blood, which clinically however cannot exceed safe, low levels. Furthermore, this method of administration lacks any tissue specificity and distributes CO throughout the entire body. Therefore, the safety and practicality of its application as an inhaled gas remains questionable.

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CO-RELEASING MOLECULES (CO-RMs): PRO-DRUGS FOR CO DELIVERY *IN VIVO*

Aiming at overcoming the above mentioned limitations on gaseous CO administration, Motterlini, Mann and colleagues proposed the use of CO-releasing molecules (CO-RMs) as pharmaceutical agents in 2002 [6]. Such molecules are meant to be administered to a living organism, a mammal in particular, and migrate to the target where CO will be delivered in a manner that elicits its therapeutic action. Thus, CO-RMs are pro-drugs that carry and deliver CO as the active principle. Ideally, CO-RMs should be able to reach their targets in the diseased tissues without having lost CO to the blood stream. In this way their activity will be maximized and correspondingly the doses decreased, which results in increased safety due to less CO spill-over and also to less pro-drug metabolites [3].

To be a CO-RM, a molecule must undergo some decomposition process *in vivo* that results in decarbonylation, or CO loss. Such decarbonylation can be spontaneous (thermal decomposition), triggered chemically or enzymatically and ideally should take place at the diseased tissue by virtue of the discriminating presence of some distinctive chemical property associated with the diseased state. Particular enzymes, pH variations and increased reactive oxygen species (ROS) concentrations are among the most attractive kinds of trigger events to start decarbonylation in a pharmacologically useful *in vivo* context. However, other possibilities cannot be discarded, e.g. light irradiation in skin diseases and other special situations [7].

Decarbonylation reactions may be found in organic and inorganic compounds. Adding possible enzymatic transformations of CO-RMs will further increase the options. In 2001, within one and half month of each other, two patent applications claimed the use of the first CO releasing molecules. Buelow and Woo disclosed the use of dichloromethane (CH_2Cl_2 (DCM)) [8], whereas Motterlini and Mann disclosed the use of transition metal carbonyls, in particular two $Ru^{II}(CO)_3$ complexes known as CORM-2 and CORM-3 [9]. Shortly thereafter, some of us disclosed a wider range of CO-RMs which included organic molecules like tertiary aldehydes, oxalates, the inorganic boranocarbonate $Na_2[H_3BCO_2]$ (CORM-A1) besides several other families of transition metal complexes (Fig. (**1**)) [10].

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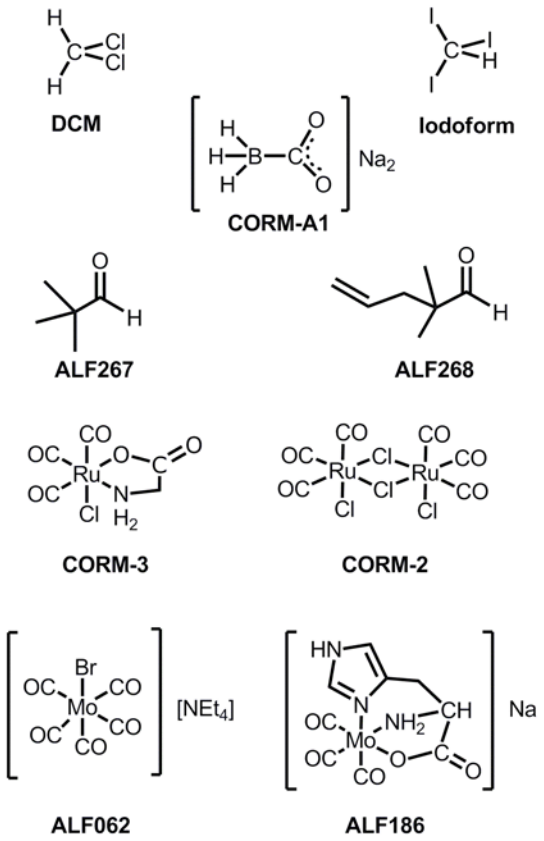

Fig. (1). Biologically tested CO-RMs: CO-RMs that have been used in animal models of disease and/or as bactericides.

In the case of DCM the release of CO *in vivo* is effected by the cytochrome P-450 oxidative system which decomposes DCM into CO, CO_2 and HCl [8]. The presence of CO was confirmed by the dose dependent elevation of COHb levels in circulation. A range of biological data presented by these authors confirmed the therapeutic efficacy of CO. The use of the related iodoform was later claimed to be effective in the treatment of rheumatoid arthritis (RA) [11].

The sodium boranocarbonate, $Na_2[H_3BCO_2]$, which had once been claimed to have anti-inflammatory and other pharmacological properties [12, 13], was shown to be a very well behaved CO-RM that releases CO in response to pH changes [14]. This compound, also known as CORM-A1, has produced interesting therapeutic data in several animal models of disease including recently in experimental allergic encephalomyelitis (EAE) in mice [15].

Some tertiary aldehydes that were shown to have beneficial effects in the Adjuvant Induced Arthritis (AIA) rat model of RA, release CO in response to the presence of ROS like peroxides and hydroperoxides [16].

However, the results in the literature [17], and more so, our work over the last eight years and *ca.* 800 compounds, has shown that transition metal carbonyls are the most reliable and flexible source of CO-RMs. The already mentioned CORM-2 ($[Ru(CO)_3Cl_2]_2$) and CORM-3 ($[fac\text{-}Ru(CO)_3Cl(\kappa^2\text{-}H_2NCH_2CO_2)]$) were among the first CO-RMs to show effective biological and therapeutic activity in a variety of *in vitro*, *ex-vivo* and *in vivo* studies, including bactericidal activity [18, 19]. They are, to this day, the most widely studied CO-RMs and their biological *in vivo* efficacy has been recently reviewed in detail by two of the leading authors in the field of biomedical research on CO [3]. Air-stable and water-soluble CORM-3 [20], attracted wide interest because of its activity in animal models of diseases with major clinical indications, such as transplantation [21], myocardial infarction [22], and RA [23]. One of the most attractive, yet intriguing features of the use of CORM-3 in mammals is the fact that it doesn't increase the blood levels of COHb much above baseline at therapeutic doses. We will come back to this topic below.

METAL CARBONYL COMPLEXES IN BIOLOGY

Metal carbonyl complexes (MCCs) are central entities in the field of organo-transition metal chemistry. Their general formula $M(CO)_xL_y$ indicates that the metal is coordinated by x CO ligands and by y C, N, O, S, P or halide ligands. Two properties rapidly distinguish these complexes from the general classical coordination compounds: i) the metal is in a low or very low oxidation state; ii) the total number of valence electrons in the coordination sphere of the metal is normally 18, except for group 4, 9 and 10 where the 16-electron count is very common.

The first property means that oxidation weakens the M-CO bond and leads to CO release. The second property means that most MCCs are kinetically inert with strong covalent bonds that are not easily mobilized in ligand substitution reactions. On the contrary, MCCs are readily activated by irradiation with UV or visible light to release CO. MCCs are not very common in biology but are readily formed by reaction of CO with reduced hemeproteins like myoglobin or hemoglobin. However, as already predicted by the first property mentioned above, oxidation of the heme to Fe(III) leads to loss of CO. In fact, this property is associated with a low stability of MCCs under oxic, atmospheric conditions: upon oxidation MCCs usually decompose and release CO. Thermal replacement or substitution of CO ligands in the dark or under anaerobic conditions is normally limited to situations where the metal is already not very electron rich or oxidized (weak overall M-CO bonds) or when one of the ancillary ligands assists CO substitution by stabilization of the reaction intermediate 16 e^- species. Such assistance may happen when the ancillary ligand is a π-donor, like RO^-, or Cl^-. For instance, the complex $Mn(CO)_5X$ loses one CO ligand rather readily to one entering nucleophile when X = Cl, Br or NCO (all π-donors) but not when X = H or CH_3 (sigma donors) [24].

The fact that the σ-donor orbital of CO is anti-bonding means that in M-CO the C atom is more positive than in the free CO molecule and the C≡O bond is reinforced. In this situation the C atom of the bound CO is activated and can react with incoming nucleophiles. This is the basis of the water-gas-shift reaction whereby the metal catalyzed reaction of CO with H_2O leads to CO_2 and H_2. Some metals, and more generally, cationic MCCs are prone to undergo this reaction which depletes the CO load of the complex. The $[Ru^{II}(CO)_3L_3]^{0/z+}$ complexes are well known to undergo this type of reaction.

Despite the astonishing progress of MCC chemistry throughout the XX^{th} century, its avenues never crossed biology or biochemistry fields until rather recently. On the one hand, MCC chemistry grew hand-in-hand with industrial hydrocarbon and petrochemical catalysis and technology. On the other hand there may have been a subconscious awareness of the extreme toxicity of MCCs like $Ni(CO)_4$ which was assumed as the default of all other MCCs and their whole chemical space. The example of the coordination of CO to heme was mainly valued as a reference to CO toxicity. However, the discovery of Fe-CO and Ni-CO bonds at the active sites of hydrogenases (see ref 33 and references cited therein) and CO dehydrogenase [25], showed that Nature actually could make good use of one of its primeaval gasesous molecules. Initial shy advances showed that not all MCCs were unstable to water or oxygen [26]. The impressive stability of the fragment $[M(CO)_3]^+$ (M = Mn, Tc, Re) led to the development of a very elegant and rich field of radiopharmaceutical applications based on Tc imaging [27]. Although MCCs seemed little adapted to biology, bio-organometallic chemistry slowly emerged as a new discipline where CO played the role of

an inert spectator or reporter ligand [28]. Almost in parallel, the biologic and therapeutic activity of CO started to be disclosed and the conditions were met to bring CO and its complexes into the realm of the pharmacological development and of metal based drug development.

TRANSLATE MCCS INTO PHARMACEUTICALLY USEFUL DRUGS

In order to become pharmaceutically acceptable a CO-RM must: i) have sufficient solubility in aqueous solutions; ii) be active and potent; iii) have a good safety profile; iv) have a reasonable pharmacokinetic (PK) profile *in vivo*; v) be stable for administration but transformable *in vivo* to release CO and non-toxic metabolites.

The construction of such entities requires the choice of: 1) the metal; 2) the number of its CO ligands; 3) the nature of the ancillary ligands. The first choice may substantially dictate the toxicity profile of the CO-RM and its metabolites. The third choice is of paramount importance because such ancillary ligands will not only determine the oxidative and substitutional stability of the MCC but will also control its solubility, biocompatibility, biodistribution and other pharmacokinetic parameters. The second choice is mainly conditioned by the profile of CO delivery that is required for a given therapeutic application and also by the toxicity of CO itself.

Since metal carbonyl chemistry was developed in the absence (and usually in the absolute exclusion of) air and water, few data are available on the reactivity of MCCs toward the kind of molecules that they have to encounter under oxic, aqueous physiological conditions. Experience shows that the presence of several CO ligands on a given coordination sphere strongly increases the lipophilic character of the complex turning water solubility into a main hurdle. This is usually overcome by preparing charged complexes or by decorating the ancillary ligands with water-compatible and solubility-enhancing functions selected from the medicinal chemistry repository.

Quite surprinsingly recent experiences also show that a vast majority of MCCs are actually rather air stable in aqueous aerobic conditions and liberate CO at relatively slow and controllable rates [29]. Of course, manipulation of the nature of the ligands and the oxidation state of the metal may also help control oxidative stability.

Once equipped with favorable solubility and stability characteristics, a CO-RM candidate must still survive its transport in the blood stream, otherwise CO will be rapidly scavenged by hemoglobin and exhaled. Stability in the blood stream depends on the interaction with cells, such as red blood cells, leukocytes and endothelial cells, and plasma proteins, namely albumin and transferrin. If this interaction is strong, it may lead to rapid decomposition and uncontrolled release of CO in the blood stream. If it is weak, it may be beneficial and result in a longer circulation half-life of the pro-drug, which may allow the use of a lower dose and thus increase the overall safety. To avoid cardiovascular problems and toxicity COHb levels generated *in vivo* from CO-RMs (or gas) should not exceed 10% COHb which is considered a non-symptomatic level. Little is known about how MCCs or CO-RMs interact with plasma or heme proteins but experience shows that the effects can be quite striking. For instance, the biologically active complex $[Mo(CO)_3$(histidinato)]Na (ALF186) releases its CO quantitatively in blood *in vitro* within the time of mixing whereas it takes over 2h to achieve the same in aerobic phosphate-buffered saline (PBS, pH 7.4) or RPMI (Roswell Park Memorial Institute) cell culture medium [29]. When administered intraperitoneally (i.p.) to mice this compound elicits a very fast rise of COHb in circulation which peaks within *ca.* 10 min and can be accurately and reproducibly predicted from the administered dose. On the contrary, bovine serum albumin (BSA) stabilizes and modulates the decay of another experimental bioactive CO-RM, $[Mo(CO)_5Br][NEt_4]$ (ALF062) [29].

Still related to the interaction of CO-RMs with proteins is the fact that the only biological targets known for CO are hemes within hemeproteins. It is often proposed that the most effective way of achieving therapeutic CO delivery from a CO-RM requires the direct transfer (or donation) of CO from the CO-RM to a still unidentified target hemeprotein(s). This type of transfer which has played a central role in the identification and selection of CO-RMs can happen rather efficiently, for instance between myoglobin (or hemoglobin) and $Ru^{II}(CO)_3$ complexes like CORM-2 and CORM-3, by a mechanism that is completely unclear (see below) [29-31].

These facts strongly indicate the need to understand the nature of the interaction of CO-RMs with proteins since they are one of the most important factors to modulate their drug-likeliness and *in vivo* efficacy.

The study of these interactions may be carried out under quite different yet complementary approaches, ranging from chemical analysis to computational modelling of protein/CO-RM adducts or aggregates. In the following we outline the results of the structural approach that we and others have carried out using X-ray crystallography on conjugates formed between proteins and model MCCs including the very prominent, therapeutically effective CORM-3.

STRUCTURAL CHARACTERIZATION OF MCC-PROTEIN ADDUCTS

One of the most useful techniques to evaluate the interaction between proteins and MCCs is infrared (IR or FTIR) spectroscopy due to the very strong CO stretching vibrations typical of MCCs. Such CO stretching frequencies can be determined unequivocally because they appear in an otherwise signal free region of the IR spectrum, providing valuable information on the geometry of the metal carbonyl fragment, $M(CO)_x$, which remains attached to the protein. However, the method does not provide information on the bonding between such $M(CO)_x$ fragment and the protein.

One of the most solid and informative methods for this purpose is the structural characterization by X-ray diffraction cristallography of the adduct that is formed in the reaction between both species. This study requires the formation of a stable adduct in a solid cristalline form that aptly diffracts X-rays. Such stable adducts of metal carbonyls are more likely to be found when a stable metal carbonyl fragment undergoes a facile substitution reaction with a nucleophile residue from the protein. As we mentioned above [*fac*-$M(CO)_3]^+$ fragments are rather stable and are therefore amenable to structural studies by atomic resolution X-ray crystallography. Indeed, the fist successful attempt of this kind was reported by H. B. Gray and coworkers who reacted $[Re(CO)_3(phen)(H_2O)]^+$ with azurin from *Pseudomonas aeruginosa*, for electron tunneling studies. The reported structures of the adducts between the fragment $[Re(CO)_3(phen)]^+$ and the protein are formed through the coordination to several solvent exposed histidine residues [32].

Taking advantage of the fact that hen egg white lysozyme (HEWL) is a reference protein due to its ability to crystallize in a short period of time, giving robust and well diffracting crystals, Fontecave and coworkers reacted it with the tris-aqua-tricarbonylmanganese cation $[Mn(CO)_3(H_2O)_3]^{+\cdot}$ obtaining an adduct of the fragment ($[Mn(CO)_3(H_2O)_2]^+$) coordinated onto the protein by the histidine15 residue [33]. This result was certified by X-ray diffraction cristallography. As expected, IR spectroscopy gave CO stretching bands in agreement with the pattern expected for the [*fac*-$Mn(CO)_3]^+$ fragment. Following the same strategy, Binkley *et al*, have studied the interaction between HEWL and $[Re(CO)_3(H_2O)_3]^+$ with similar results. In this case, Re was used as a surrogate of ^{99m}Tc suitable for radiopharmacology and imaging [34].

These results reveal a simple pattern in which the labile H_2O ligand bound to the $[fac\text{-}M(CO)_3]^+$ fragments (M = Mn, Re) is readily replaced by histidine nucleophiles present on the surface of proteins. The stability of these metal carbonyl fragments, which doesn't qualify them as CO-RMs since they do not release CO, makes their reactivity quite predictable. Nevertheless, we thought that the principle seemed solid enough to be applied to real CO-RMs like CORM-3 which shows a marked lability in aqueous solution [20].

Before entering the discussion of this topic we think worth mentioning that the groups of G. Jaouen and Grotjahn have prepared many metal carbonyl complexes conjugated to several proteins namely with the objective of probing their utility as protein markers. For this purpose, such complexes were appropriately equipped with functional groups that undergo fast conjugation with protein residues. None of such conjugates has either been used as a CO-RM or structurally characterized (see for instance [35] and refs cited therein)

CORM-3 INTERACTION WITH PROTEINS

CORM-3 has been tested extensively for possible therapeutic applications in various animal models of disease. During circulation *in vivo*, CORM-3 has the potential to interact with a large number of proteins, namely those in the blood plasma, and most likely with two of the most abundant hemeproteins in the body, hemoglobin and myoglobin. CORM-3 $[fac\text{-}Ru(CO)_3Cl(\kappa^2\text{-}H_2NCH_2CO_2)]$ possesses two labile ancillary ligands (Cl- and glycinate) which are prone to substitution in aqueous media [20]. It is therefore reasonable to expect that this lability allows for the chemical interaction between CORM-3 and proteins in the blood. To probe such interactions, four different techniques were used: Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES), X-ray crystallography, Liquid-Chromatography Mass Spectrometry (LC-MS) and IR.

As a fast method to detect the presence of covalent bonds between proteins and CORM-3, protein samples incubated with the compound were separated by dialysis and its Ru contents analyzed by ICP-AES. Human albumin, human transferrin, horse heart myoglobin, human hemoglobin, as well as the model protein HEWL gave positive results in Ru detection confirming the existance of some strong interaction, most likely covalent interactions.

Although X-ray crystallography was attempted for the adducts of CORM-3 with all the proteins, in a broad variety of conditions, suitable crystals for diffraction were found only for HEWL. Soaking experiments were performed using a wide range of concentrations of CORM-3 with different periods of soaking. The best results were obtained when HEWL crystals were stabilized in mother liquor containing 12% NaCl (harvesting buffer - HB) and soaked in CORM-3 also dissolved in HB, to a final concentration of 100 mM. Data was collected (ID14-I, ESRf, Grenoble) up to 1.67 Å resolution. The crystal structure, recently published, shows a $Ru(CO)_2$ rather than a $Ru(CO)_3$ fragment binding to the protein (Fig. (**2**)) [31]. Binding at His15, with occupancy of 0.8, Ru adopts an octahedral geometry with the Nε2 of histidine side chain, two CO molecules, and three water molecules. Upon protein-CORM adduct formation, the two ancillary ligands, together with one carbonyl are lost, and the vacant coordination positions are filled with the side chains of the protein residues and water molecules. The absence of the chloride ion, present in CORM-3, in this protein-Ru adduct was ascertained by (1) B-factor analysis and (2) by inspection of anomalous electron density maps. Two more sites were found in solvent exposed residues at the surface of the protein, Asp18 and Asp52. The electron density is not conclusive at those Ru sites, due to lower Ru occupancies: 0.5 for the Asp18 site and 0.4 for the Asp52

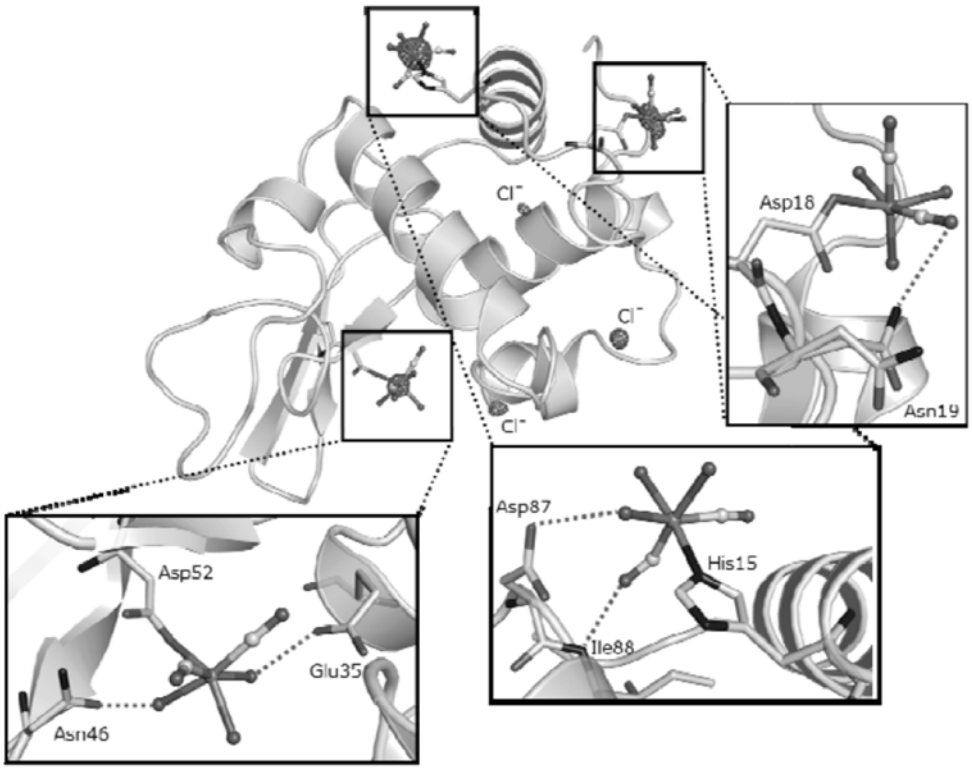

Fig. (2). Overall structure of HEWL bound to three CORM-3 moieties. Geometry and coordination of each binding site in the protein-CORM-3 adduct is shown in the detail and hydrogen bonds between the complex and protein residues are depicted in dashed lines. The three chloride ions found in the structure are also represented, together with the anomalous electron density maps, contoured at 3.0 σ.

site. Therefore, these two protein-CORM-3 adducts cannot be modeled as accurately as the His15 adduct, which is reflected in the larger B factors of the metal ligands. For the three sites, CO ligands are found at equatorial positions in a linear and bent mode.

The most important point in all this structural information is the loss of one CO ligand in the adduct. This loss seems to be a rather facile and rapid process, because LC-MS analysis of reaction mixtures of CORM-3 and protein in different ratios (1 or 10 equiv) incubated in water at room temperature for 10 min, all showed a single metal-protein adduct corresponding to the addition of one unit of $Ru^{II}(CO)_2$ (157 *m/z*) to lysozyme [31].

Although we were unable to obtain X-ray crystallographic data on the adducts of CORM-3 with the other proteins we still could show that all other conjugates also contain a $Ru(CO)_2$ fragment. Indeed, the FTIR spectra of all the conjugates of CORM-3 obtained and purified by dialysis and then lyophilized, show two pairs of CO stretching vibrations assignable to *cis*-$Ru^{II}(CO)_2$ fragments: one with peaks at 2056 cm^{-1} and 1981 cm^{-1} and another with peaks around 2038 cm^{-1} and 1950 cm^{-1}. The former pair of vibrations has the same wave numbers that are observed in the FTIR spectrum of the X-ray diffracting crystals of the adduct *cis*-$[Ru^{II}(CO)_2(H_2O)_3(His15)]^{2+}$ identified in Fig. (**2**). The other pair of vibrations at lower energies is tentatively assigned to the *cis*-$Ru^{II}(CO)_2$ fragments in the two lower occupancy sites.

Altogether, these data clearly show that the $Ru(CO)_3$ fragment in CORM-3 is not stable enough to survive the interaction with a number of proteins in intact form and rapidly loses one CO. The importance of this fact is crucial for interpreting its mode of action in terms of CO delivery and has to be evaluated within the more general set of CO release data already gathered for CORM-3.

CO DELIVERY FROM CORM-3

The classification of CORM-3, [*fac*-$Ru(CO)_3Cl(\kappa^2$-$H_2NCH_2CO_2)$], as a fast CO releaser was based on its ability to rapidly carbonylate deoxy-myoglobin, at ≈ 30-50 μM concentrations, to form COMb. Only one of its three CO ligands is transferred. It is also known that leaving CORM-3 in buffer solution at physiologic pH for *ca.* 24 h produces a solution of the so-called i-CORM-3 (inactivated CORM-3) which is not able to carbonylate Mb or does it only very slowly. The difference between many positive biological effects registered upon treatment with CORM-3 solutions and their absence in the respective i-CORM-3 treated controls has been the rationale for attributing those biological effects to CO.

It is quite tempting to assume that the CO ligand, lost in the transformation of CORM-3 to i-CORM-3, is simply removed from CORM-3 by ligand substitution and escapes into the headspace. However, GC monitoring of the headspace of closed vials containing solutions of CORM-3 in H_2O, saline, PBS, (pH 7.4), RPMI cell growth medium in the presence or absence of fetal bovine serum (FBS) never detected CO gas even with the RCP detector sensitive to 1 digit ppb levels of CO gas. Instead, about one molar equivalent of CO_2 is formed after 24 hours (GC-TCD detection). Since no CO gas is released when CORM-3 is reacted with solutions of FBS we conclude that the one CO ligand lost in the interaction between CORM-3 and the proteins described above, also leaves as CO_2 which is indeed detected.

These data confirm previous disclosures by Motterlini, Mann and coworkers [20]. On the one hand they have shown that when dissolved in water, CORM-3 immediately undergoes a water-gas-shift reaction whereby one CO ligand reacts with H_2O to form a metallocarboxylate which releases H^+ to the solution. Lowering the pH well below the physiological range, pH < 3, reverts the latter species towards the $Ru(CO)_3$ fragment [20]. They also showed that once dissolved in plasma CORM-3 has a half-life of 3.6 min well below the corresponding values of 98.5 h in H_2O and 20.4 min in PBS at pH 7.4 [36].

Altogether, these data strongly suggest that plasma proteins which do not have heme groups, accelerate the decomposition of CORM-3 liberating CO_2 and covalently binding *cis*-$Ru^{II}(CO)_2$ fragments. However, the reaction with myoglobin (and certainly also with hemoglobin) has a diferent course since one of the three CO ligands is rapidly transferred to the heme. The remaining a *cis*-$Ru^{II}(CO)_2$ fragment stays also bound to the protein as we have shown by FTIR [31].

The question then remains: how does CORM-3 elicit its therapeutic activity? When mixed with blood *in vitro*, or once it is administered to a mammal at therapeutic doses, CORM-3 is not hemolytic and does not raise the levels of COHb in circulation much above basal level. In other words it doesn't transfer CO to blood. However, it is not obvious at all how it will survive intact on the way to reach other heme proteins inside cells and tissues given the fast CO depleting reaction it undergoes with the rather abundant plasma proteins in circulation.

The answer to such question becomes even more difficult to find since the real target(s) for CO is (are) not yet known, and therefore, the regimen (rate, dose and place) of CO delivery to such target that is necessary to start the therapeutic cascade is also unknown. Nevertheless we would like to put forward an alternative explanation based on the fate of the *cis*-$Ru^{II}(CO)_2$ adducts that should be readily formed after CORM-3 administration *in vivo*. Once formed, such CO containing protein adducts will remain in circulation while slowly decaying. In this process they can produce sustained, prolonged CO release in amounts that are adequate to elicit a therapeutic response but small enough as to not increase COHb to toxic levels. Quite remarkably, this kind of pharmacological profile was recently reported by Prabhu and coworkers [22] who showed that CORM-3 alleviates post-infarction left ventricular remodeling and apoptosis following 24 days of daily *in vivo* i.p. administration of a 40 mg/kg dose in mice. During this time span a fairly constant COHb value of 6% was maintained. Slow, steady CO release from plasma-$Ru(CO)_2^{2+}$ species seems conceivably the simplest way to achieve this low constant COHb level. Such kind of plasma protein control of the therapeutic activity of a metal based drug precursor, although still unpredictable has ample precedent and rationale in the literature.[37, 38]

FINAL REMARKS

The present overview is not intended to be an exhaustive account of the field of CO-based therapy through CO-RMs as molecular vectors for its delivery. It simply intends to highlight the motivations and reasonings behind the flow of studies which are actively attempting to realize the therapeutic potential of CO, overcoming its limitations as a gaseous drug by designing therapeutically useful CO-RMs. Although a large variety of such pro-drugs has been prepared and patented recently featuring different transition metals such as, Fe [39-43], Co [44], and Re [45], together with different ancillary ligands and other molecular devices [46], none of the published compounds has been shown to exhibit pharmacologic properties that allow its use as a drug. Understanding the interactions of CO-RMs with plasma proteins or oxygen carriers is just one of the important steps on the way to designing CO-RMs with such characteristics. The first results in this field are now reported and help further clarify the behavior of one of the most useful experimental CO-RMs produced so far, CORM-3. Similar studies on other transition metal based CO-releasers are warranted in order to clarify the chemistry of CO delivery *in vivo* and guide the search for pharmacologically acceptable, potent, drug-like CO-RMs for CO based therapy.

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